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(54) Title: METHOD AND APPARATUS FOR DETERMINING LOCAL VISCOELASTICITY

(57) Abstract: Disclosed is an apparatus and method for determining local viscoelasticity in a fluid or a gel. The method employs a scanning optical microscope in combination with a force manipulating function. A particle is provided within the fluid or gel in which the local viscoelasticity is to be determined. The particle is periodically modulated using the force manipulating function to set the particle in periodic motion about the focal region of the scanning optical microscope. The second harmonic of the signal from the focal region of the scanning optical microscope is determined with respect to the particle in motion. The phase delay of the second harmonic determined in this manner is used to calculate local viscoelasticity.

METHOD AND APPARATUS FOR DETERMINING LOCAL VISCOELASTICITY

Background of the Invention

In the study of colloidal suspensions, polymer solutions, and in cell and micro-biology it is often desirable to have a direct observation in real time of the changes in the local viscoelasticity which affect the dynamics of objects such as colloidal particles, cells and organelles. For example, the cells transport and diffusion of macro-molecules both depend on the local environment. It is especially important to have high spatial resolution in complex environments in which local inhomogeneities are present.

Existing methods such as localized dynamic light scattering (Bar-Ziv et al., Phys. Rev. Lett. 78: 154-157 (1997)) require a complex setup. In most methods, determining the stiffness of a trapping beam requires a well-calibrated position detector (Visscher et al., Selected topics in quantum electronics 2: 1066-1076 (1996)). Determining the characteristic time of the motion is done through a cumbersome fit calculation to the power spectrum of the position, which requires substantial time to collect. Digital video microscopy (Crocker et al., Phys. Rev. Lett. 82: 4352-4355 (1998)) is a good tool to study the dynamics but requires the processing of many digitized video images, which can not be done in real time and is often complicated by the image quality. A method which can be used to measure local changes in viscoelasticity at high spatial resolution and high temporal resolution would represent a significant advance in the art.

Summary of the Invention

The present invention relates, in one aspect, to an apparatus and method for determining local viscoelasticity in a fluid or a gel. The method employs a scanning optical microscope in combination with a force manipulating function. A particle is provided within the fluid or gel in which the local viscoelasticity determination is to be made. The particle is periodically modulated using the force manipulating function to set the particle in periodic motion about the focal region of the scanning optical microscope. The second harmonic of the signal from the focal region of the scanning optical microscope is determined with respect to the particle in motion. The phase delay of the second harmonic determined in this manner is used to calculate local viscosity.

Brief Description of the Drawings

Fig. 1 is a diagrammatic representation of a confocal embodiment of the present invention.

Fig. 2 is a diagrammatic representation of a confocal embodiment of the type employed in the experiments described in the Exemplification section set forth below.

Fig. 3 is a diagrammatic representation of measurements made at a constant laser power of 1.3 mw.

Detailed Description of the Invention

The present invention relates to a method for determining local viscoelasticity in a fluid or a gel. This invention finds application in a variety of fields including, for example, biological and chemical applications.

With respect to biological application, in preferred embodiments, the fluid is a biological fluid (i.e., a fluid produced by an organism), or a non-biological fluid which contains biological cells or biomolecules (i.e., molecules produced by an organism). A biological fluid may be an intracellular fluid, such as cytosol. Alternatively, a biological fluid may be an extracellular fluid such as serum. Examples of non-biological fluids which contain biological cells or biomolecules include cell culture medium, buffered saline solution, tissue culture medium, etc.

The apparatus used to determine local viscoelasticity in the method of the present invention is comprised of multiple elements. A scanning optical microscope provides the ability to probe the local environment in a fast and sensitive manner. Examples of scanning optical microscope formats useful in connection with the present invention include confocal microscopes as well as two photon microscopes.

A force manipulating function is also provided in connection with the methods of the present invention. The force manipulating function is used to sequester and manipulate a particle within the fluid or gel of interest. Examples of suitable force manipulating functions include, for example, photon force manipulating functions (e.g., laser trap or optical tweezers) and magnetic force manipulating functions. With respect to the former, the laser is preferably tuned to the infrared spectrum.

To determine local viscoelasticity, it is necessary to provide a particle which is suitable for manipulation in the fluid or gel using the force manipulating function. The required properties of the particle for use in connection with the methods of the invention vary depending upon the nature of the force manipulating function. For example, when using a laser trap as a force manipulating function, the particle must be transparent and have a refractive index higher than that of the surrounding medium. When using a magnetic force manipulating function, the particle must be a magnetic particle. It should be noted that suitable particles for use in connection with the present invention may be naturally ocurring in the fluid or gel of interest. For example, it is a matter or routine experimentation to determine whether an intracellular organelle, such as mitochondria or chloroplasts, can be visualized, trapped and set in motion using the force generating function of the present invention.

Once sequestered using the force generating function of the present invention, the particle is set into periodic motion by modulating the force generating function with an oscillation amplitude that is slightly larger than the diameter of the particle of interest. The resulting particle motion is periodic with a frequency equal to the driving frequency and has a phase lag because of the drag.

The second harmonic of the signal from the focal region of the scanning optical microscope is determined with respect to the particle in motion. The phase delay of this second harmonic is then determined with respect to the second harmonic of the period modulation of the force function. Such determinations are made, for example, using a phase sensitive detector. These values are then used to determine local viscoelasticity. Given the present disclosure, one of skill in the art could derive and solve local viscoelasticity equations without the use of undue experimentation. Many sets of equations could be derived to determine local viscoelasticity depending, for example, upon the degree of precision required. It is not possible within the context of a patent application to set forth all of the many possible variations which ultimately will yield a value for local viscoelasticity.

Following is an illustration of a solution route for a variant of a photonic force microscope in which confocal detection is employed. This simplifies alignment, improves stability and allows scanning imaging. In confocal detection we receive a signal from the

bead whenever it is in the focal region of the trapping beam. Therefore, in typical applications in which the bead is moving about in the trap, it is necessary to have a model to indicate when the position x of the particle is the same as the position p of the trap. In the following, we develop a mathematical model for $u = x - p$ and expect a signal whenever $u = 0$.

In optical tweezers, particles are trapped by the force exerted by the electric field of the trapping beam on the dipole moment induced in the trapped particle. The dipole moment μ induced by the optical field is given by

$$\mu = \alpha E$$

where α is the polarizability of the trapped particle. For particles much smaller than the wavelength of light α is given by

$$\alpha = 3\varepsilon_1 \frac{\varepsilon_2 - \varepsilon_1}{\varepsilon_2 + 2\varepsilon_1} V$$

V is the particle volume, ε_2 the particle dielectric constant and ε_1 the dielectric constant of the host medium. In one dimension the force on the particle is

$$F = -\mu \frac{dE}{dx} = -\frac{\alpha}{2} \frac{d|E|^2}{dx}$$

so that the particle finds itself in a potential

$$U = \frac{\alpha}{2} |E|^2$$

The motion of a micron-sized particle in common fluids takes place at small Reynolds number and viscous drag dominates inertial forces. The corresponding equation of motion of the particle in the trap, ignoring Brownian motion for the moment is

$$\gamma \frac{dx}{dt} + F = 0$$

where γ is the hydrodynamic drag coefficient. For a sphere far from any surface γ equals $6\pi\eta a$ where a is the radius of the sphere and η is the dynamic viscosity. Suppose the optical electric field of the trap is Gaussian, as occurs in a diffraction-limited beam. Then the field amplitude is proportional to $\exp(-x^2/2\sigma^2)$ so that the equation of motion is

$$\gamma \frac{dx}{dt} + \alpha(x - p(t)) \exp\left(-\frac{(x - p(t))^2}{2\sigma^2} \right) = 0 .$$

If the tweezer is set into sinusoidal motion in a fluid flowing with velocity v then

$$p(t) = vt + a\sin\omega t.$$

The corresponding equation for u is

$$\gamma\frac{du}{dt} + \alpha u\exp\left(-\frac{u^2}{2\sigma^2}\right) = -\gamma(v + a\cos\omega t)$$

These differential equations can be readily solved using the Runge-Kutta method, or can be approximated by linear differential equations with constant coefficients if the experimental conditions are such that the particle stays near the center of the trap and a parabolic approximation can be made for the particle potential.

It is convenient to recast the equation of motion into dimensionless form

$$\frac{dU}{dT} + CU\exp\left(-\frac{U^2}{2}\right) = -V - A\cos T$$

where $U = u/\sigma$ is the relative bead position in units of σ, $T=\omega t$ is the normalized time, and $A=a/\sigma$ is the oscillation amplitude of the trap in units of beam width σ and V is a normalized velocity. In the linear case this equation reduces to

$$\frac{dU}{dT} + CU = -V - A\cos T$$

whose solution is

$$U(T) = -\frac{V}{C} - \frac{A(C\cos T + \sin T)}{1 + C^2}$$

Zero crossings occur at

$$\sin T = \left(\frac{C^2}{1+C^2} - \frac{V^2}{A^2}\right)^{1/2} \pm \frac{V}{AC}$$

For the simple case $V=0$, the second harmonic phase is then

$$\varphi_2 = 2\tan^{-1} C$$

Local viscoelasticity determinations of the type described above find application in a variety of biological contexts. When cells produce biological materials such as proteins the viscosity of the fluid around and within the cells changes. The greater the change, the greater the concentration of product. Disclosed is a novel scanning probe microscope capable of forming spatial images of viscosity distributions to allow, for example, selection of overproducers.

In a preferred embodiment, the instrument combines optical tweezers with confocal optical detection. In an optical tweezer the trapped particle appears as an object in the focal point of the laser beam. If the particle moves about in the trap, the signal detected confocally from it will vary. If the tweezers are set into slight sinusoidal spatial oscillation then the position of the particle in the trap can be accurately monitored by lock-in detection of the confocal signal. In regions of higher viscosity, the trapped particle will lag further in phase behind the oscillating beam. Such an instrument can also be used to map fluid flow distributions. In regions of faster flow, the particle will be further pushed to one side of the oscillating beam. This offset too can be monitored by confocal detection. This method of microscopy will address the need in biology and medicine for real-time visualization of fluid parameters in cellular systems such as cultures. Typical applications are:

- Selection of overproducers of bioproducts such as polysaccharides (eg Pullulan). Cells secrete these polysaccharides in a halo around their bodies. A real time map of the spatial extent of the viscous polysaccharide distribution would enable real-time screening.
- Selection of hybridomas from a background population of precursor cancer cells. Hybridomas are efficient antibody factories.
- Selection of secretory mutants for protein expression. One problem with protein expression in bacteria is that the product is often left inaccessible inside the bacteria, or is hindered by a toxic buildup of the expression product inside the cell.
- Analysis of mass transfer of gasses in cell populations.
- Cell sorting by refractive index signature. The trapping strength of the optical tweezers in the microscope increases with the refractive index of the trapped particles. Sporulating bacteria can be used to make antibiotics. The microscope could select these by the high refractive index of their spore bodies.
- Differentiation of live cells from dead cells, by examining differences in their extracellular environment.

Cell interiors can be explored by using the microscope to manipulate intracellular particles such as mitochondria and chloroplasts (especially important for selection of

plant cell lines). Refinements of the present invention include variants involving fluorescent microscopy and dielectric and magnetic particles. The present invention relates also to the three dimensional case with traps of Gaussian rather than quadratic profile, and include corrections due to the fact that the beads in use actually have radii too large to use in the Rayleigh (small bead) limit.

Even a continuous wave laser can elicit two-photon fluorescence in appropriately labeled beads. The fluorescence intensity is highest when the particle is at the center of the trap, so the method of second harmonic lock-in detection is also useful in this context. One significant advantage of two-photon fluorescence is that visible fluorescence can be excited with minimally damaging near infrared light. If regular single photon excitation were employed, the tweezer wavelength would have to be in the visible, where biological materials show considerable absorption and photodamage. It is true that a weak visible exciter beam could be made collinear with an infrared trapping beam but this will come at substantial alignment cost and increased system complexity. Another significant advantage is that two-photon fluorescence is proportional to the square of the intensity. This localizes the fluorescence to the focal volume of the trap, and eliminates the need for a physical pinhole in the confocal detection unit.

Magnetic beads may also be used in place of dielectric beads. In this context, a time varying magnetic field is used to force the beads into oscillation. A substantially lower laser power can be used since the laser no longer need to supply the trapping force. A feedback system would be designed to lock the probe laser to the bead under test by nulling out the fundamental signal in x, y and z, or by maximizing the average confocal signal. The second harmonic signal would give information on viscosity as described above.

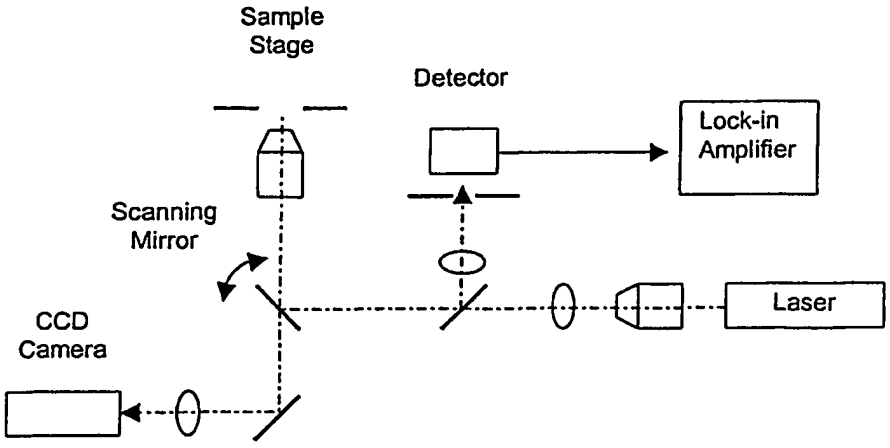

Figure 1

In one embodiment, the present invention relates to a device of the type shown in Figure 1. In this embodiment, a scanning mirror oscillates the trap back and forth and a lock-in amplifier measures the phase shift between the confocal signal and the second harmonic of the spatial oscillation frequency of the trap. At higher viscosities, this phase shift increases and as shown below a mathematical model can be developed to enable determination of the medium viscosity.

An important distinction between the present invention and prior art work is the associated use of 2nd harmonic phase detection (using for example a lock-in amplifier) to monitor the position of the probe particle relative to the center of the oscillating trap. In the usual use of lock-in detection the signal is detected at the same frequency (fundamental) as the sinusoidal oscillation of the stimulus. But in connection with the present invention, the bead comes into focus twice every cycle, so there will be no signal at the fundamental. Detection at twice the frequency of oscillation of the stimulus is required and, therefore, second harmonic detection is required.

Exemplification

1. Theoretical Considerations

The equation of motion of a particle in a harmonic potential is:

$$\beta\dot{x}(t) + \alpha \cdot [x(t) - p(t)] = 0 \tag{1}$$

where $x(t)$ is the particle position, $p(t)$ is the position of the center of the potential well (the focused laser beam in our case). β and α are the drag coefficient and the trap stiffness respectively. We have neglected the inertial term and the Langevin (stochastic) force.

For a potential moving in a sinusoidal fashion, this equation becomes:

$$\dot{x}(t) + \frac{x(t)}{\tau} = \frac{p_0}{\tau}\sin\omega t \tag{2}$$

where p_0 and ω are the amplitude and angular frequency of the laser motion, and τ is the characteristic time scale of the motion:

$$\tau = \frac{\beta}{\alpha} \tag{3}$$

Since the trap stiffness α is proportional to the optical power P, we get

$$\tau = \frac{\beta}{\alpha_0 P} \equiv \frac{b}{P} \tag{4}$$

where α_0 is a measure of the trapping efficiency. b is an invariant property of the combined particle-medium-optics system (independent of the laser power or the driving frequency) and has units of energy. The solution to equation 2 is:

$$x(t) = \frac{p_0}{(1+\omega^2\tau^2)^{1/2}} \cdot \sin(\omega t + \theta) \tag{5}$$

where the phase difference between the particle's position relative to the laser beam is:

$$\theta = -\tan^{-1}(\omega\tau) \tag{6}$$

Given that relation 6 holds, a measurement of θ at a given ω constitutes a measurement of τ.

2. Experimental Design

The spectrum of the back-scattered light signal from a laser beam moving with a frequency ω across a microparticle will exhibit peaks at the second harmonic since the beam traverses the particle twice in one cycle and since the confocality condition means that we detect a signal only when the focused laser beam coincides with the position of the particle.

The particle also exhibits Brownian motion, which appears as noise that hides the signal at 2ω. In order to account for that, we use a lock-in amplifier, which locks on to the second harmonic of the driving frequency by using a reference signal taken directly from the oscillating mirror. Figure 2 shows a schematic of the experimental setup. A $0.9\mu m$ diameter silica microsphere suspended in water is held in the optical tweezers and observed by a camera. 100x, NA1.25 oil immersion objective lens is used to form the trap and capture the scattered light as well as for the imaging. The laser source is an Argon ion laser operating at 488nm. The lock-in amplifier measures the phase difference between the back-scattered light signal and the reference signal at 2ω. To find the relation between the measured phase angle φ and the particle's phase lag θ, we need to look at the measurement procedure itself.

A simple position detection setup is used to generate a reliable reference signal. It consists of a He-Ne laser, which is reflected off the back side of the moving mirror to a split photodiode (see fig. 2). A difference amplifier connected to the outputs of the split photodiode produces the desired position signal of the mirror. The lock-in amplifier receives this sinusoidally varying voltage at a frequency ω as a reference. It then generates internally a pure sine wave signal at the second harmonic:

$$ref_{2\omega} = \sin(2\omega t) \tag{7}$$

The signal from the photomultiplier tube (PMT) can be written as:

$$sig = -\sin[2\omega(t - t_c) + \frac{\pi}{2}] + noise \tag{8}$$

t_c represents the times when the particle and the center of the trap coincide. We add $\pi/2$ to account for the fact that the signal is high at the coincidence times. The signal is negative due to the negative bias voltage of the PMT. Note that the lock-in detection treats contributions to the signal at other frequencies as noise.

We can rewrite the signal at 2ω:

$$sig_{2\omega} = \sin(2\omega t + \varphi) \tag{9}$$

with the phase angle φ given by:

$$\varphi = -2\omega t_c - \frac{\pi}{2} \tag{10}$$

φ is the measured quantity in our experiment. To find t_c we need to solve the equation $x(t) = p(t) = p_0 \sin\omega t$ for t. The solution is given from equation 5 by:

$$t_c = \frac{1}{\omega}\tan^{-1}(\cot(\theta)) \tag{11}$$

which gives us the relation between θ and φ:

$$\tan(\frac{\varphi + \frac{\pi}{2}}{2}) = -\cot(\theta) \tag{12}$$

Finally, using equation 6, we get the relation between φ, τ and b which forms the basis of our measurement technique:

$$\cot(\frac{\varphi + \frac{\pi}{2}}{2}) = \omega\tau = \frac{\omega b}{P} \tag{13}$$

3. Results

Figure 2 shows the results of measurements of φ at different ω's at a constant laser power of $1.3mw$ (power at the sample). Similar measurements were made at different laser powers of w=218.6 rad/sec (34.8Hz). All measurements were made on the same particle at the same distance from the cover glass $\simeq 50\mu m$. The data clearly show that relation 13 holds.

From the slope of the graph in figure 3 we get $b = 1/(slope \cdot \omega) = 2.20 \pm 0.01\mu J$. From the slope in figure 2 we get $\tau = 1.68 \pm 0.01ms$, which gives $b = \tau \cdot P = 2.18 \pm 0.02\mu J$. Each measurement of φ is a statistical average over a period of $\simeq 30sec$ and the error bars are derived from the standard deviation. The slopes were calculated using a linear regression algorithm that forces the curve to go through the origin. We further calculate $\alpha = 5.0 \cdot 10^{-3} pn/nm$ from our measurement of τ using a Stokes drag coefficient of $\beta = 6\pi\eta a = 8.48 \cdot 10^{-9} kg/s$, using the viscosity of water $\eta = 1 \cdot 10^{-3} kg/m/sec$.

4. Summary

The results reported above demonstrate a new analytical method method which combines optical tweezers with confocal detection to probe the microscopic scale environment and dynamics of microparticles. The results confirm the validity of the point particle model in an oscillating harmonic potential. While the multiple measurements taken over a range of frequencies and optical powers are necessary to validate the measurement technique, actual measurements of τ require in principle only one measurement at a given frequency and power. This is important because it allows one to monitor time variations of these properties in a time varying medium (e.g., a polymer solution undergoing a phase transition). If this method is used in conjunction with another method to get α then β can be determined absolutely.

A significant advantage of this technique relative to prior art methods is that it can be used with a relatively low laser power, since the optical tweezers does not need to trap the particle with a strong force, but rather has to exert enough force to move the particle in its direction. We note that there is a direct relationship between the range of frequencies and optical powers one should use for this measurement to succeed. For a given optical power, as the frequency of the motion is increase there is a point where the particle's response becomes too slow to follow the trap. At this point the lock frequency breaks down because Brownian motion starts to dominate. At a very low frequency of motion the particle with move together with the trap, and the lock frequency will break down because the second harmonic component of the signal will be very weak and noisy. This does not mean that one could not use this method at very high or very low frequencies, it only means that the strength of the trap has to be adjusted appropriately at these extremes. Finally, this method can be used as a new confocal imaging technique by obtaining successive measurements of τ at different locations in the sample.

CLAIMS

1. A method for determining local viscoelasticity in a fluid or gel, the method comprising:
   a) providing a scanning optical microscope;
   b) providing a force manipulating function;
   c) providing a fluid or gel in which local viscoelasticity is to be determined;
   d) providing, in the fluid or gel of step c), a particle which can be moved using the force manipulating function;
   e) periodically modulating the force manipulating function to set the particle in periodic motion about the focal region of the scanning optical microscope;
   f) determining second harmonic of the signal from the focal region of the scanning optical microscope with respect to the particle in motion of step e);
   g) determining phase delay of the second harmonic of step f) with respect to the second harmonic of the period modulation of the force function of step e);
   h) employing the phase delay variable determined in step g) to calculate local viscoelasticity in the fluid or gel.

2. The method of Claim 1 wherein the scanning optical microscope is a confocal microscope.

3. The method of Claim 1 wherein the scanning optical microscope is a multi-photon excitation microscope.

4. The method of Claim 1 wherein the force manipulating function is a photon force.

5. The method of Claim 4 wherein the photon force is a laser trap.

6. The method of Claim 5 wherein the wavelength of the laser is tuned to the infrared spectrum.

7. The method of Claim 5 wherein the light field of the scanning optical microscope and the light field of the laser trap are one in the same.

8. The method of Claim 1 wherein the force manipulating function is a magnetic force.

9. The method of Claim 1 wherein the fluid is a biological fluid.

10. The method of Claim 9 wherein the biological fluid is intracellular or extracellular.

11. The method of Claim 9 wherein the biological fluid is cytosol.

12. The method of Claim 9 wherein the biological fluid is serum.

13. The method of Claim 1 wherein the particle of step d) is naturally occurring in the fluid or gel of interest.

14. The method of Claim 13 wherein the fluid is a biofluid and the particle is an intracellular particle.

15. The method of Claim 14 wherein the intracellular particle is an organelle.

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NO PRESENTADO(A) EN EL MOMENTO DE LA PUBLICACIÓN

NON SOUMIS(E) AU MOMENT DE LA PUBLICATION

EXPERIMENTAL SETUP OF
CONFOCAL LASER TWEEZERS

BS - BEAM SPLITTER
DSM - DOUBLE SIDED MIRROR
ON A GALVENOMETER

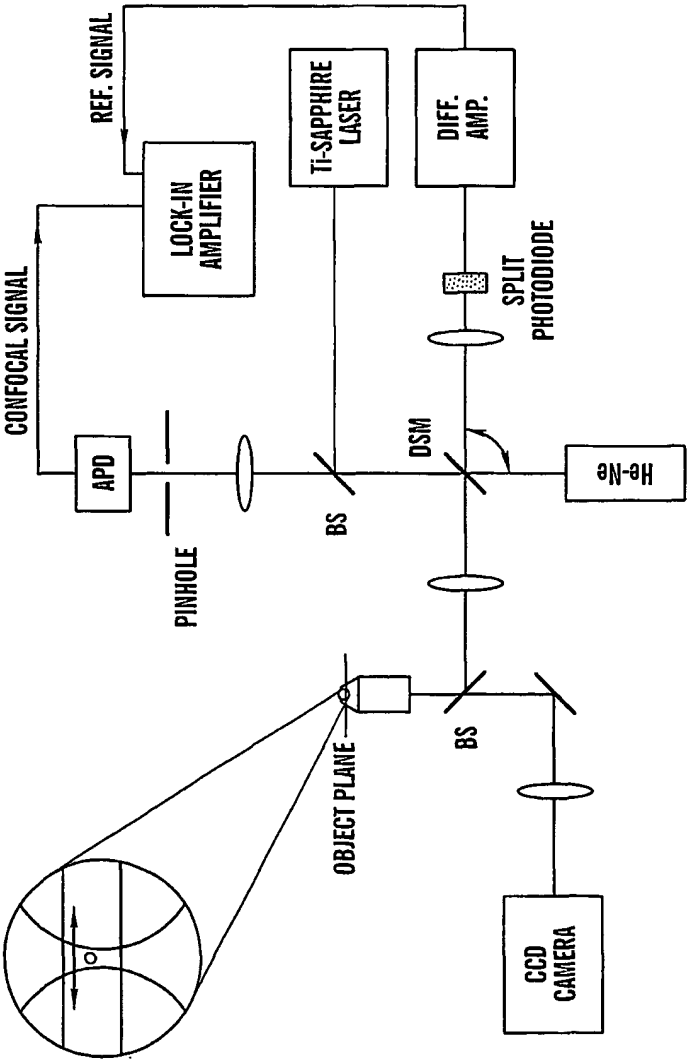

FIG. 2

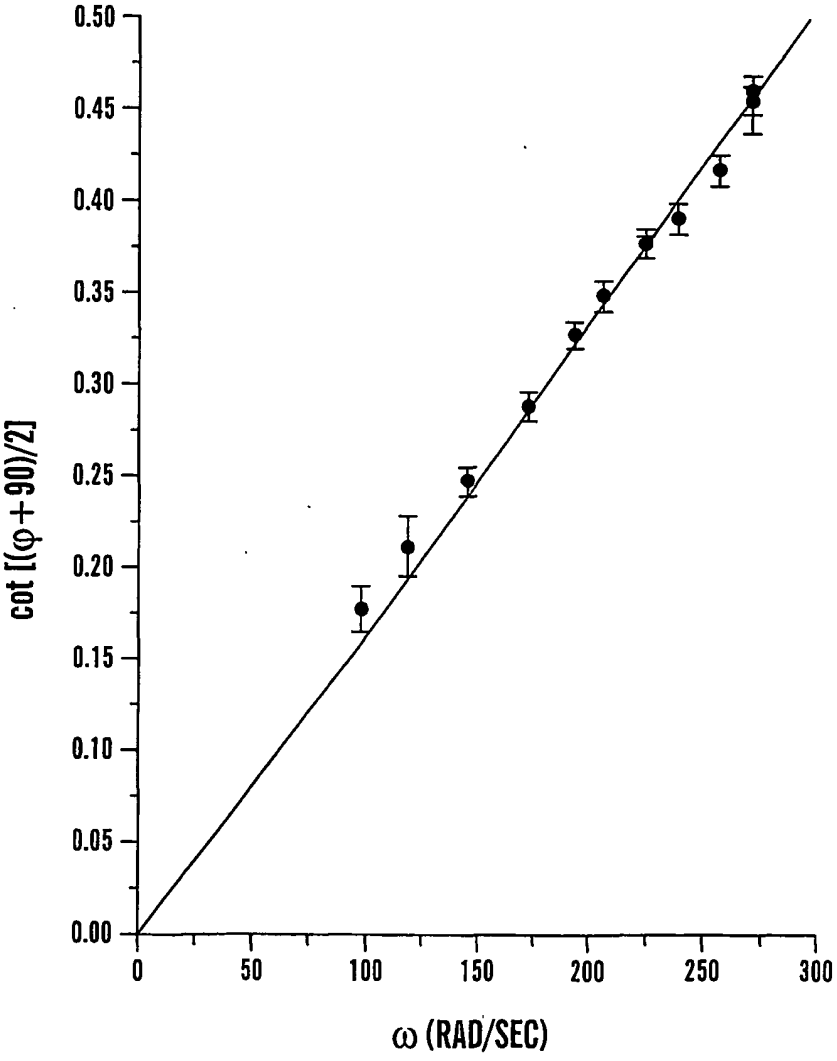
0.50
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
cot [(φ+90)/2]
0
50
100
150
200
250
300
ω (RAD/SEC)

FIG. 3

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/14729

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :G01N 11/16

US CL :436/63, 69, 164; 73/54.24, 54.25, 54.26, 54.27; 356/426, 427

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

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Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 3,861,197 A (ADLER) 21 January 1975, entire document.	1-15
A	US 5,158,720 A (LEVY) 27 October 1992, entire document.	1-15
A	US 5,707,587 A (BLANCHARD et al) 13 January 1998, entire document.	1-15
A	US 4,779,452 A (COHEN-TENOUDJI et al) 25 October 1988, entire document.	1-15
A	US 4,149,405 A (RINGROSE) 17 April 1979, entire document.	1-15
A	US 5,590,560 A (JOOS et al) 07 January 1997, entire document.	1-15

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"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 22 JUNE 2001	Date of mailing of the international search report 03 JUL 2001
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